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A GATA/RUNX *cis*-regulatory module couples *Drosophila* blood cell commitment and differentiation into crystal cells

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Abstract

Members of the RUNX and GATA transcription factor families play critical roles during hematopoiesis from *Drosophila* to mammals. In *Drosophila*, the formation of the crystal cell hematopoietic lineage depends on the continuous expression of the lineage-specific RUNX factor Lozenge (Lz) and on its interaction with the GATA factor Serpent (Srp). Crystal cells are the main source of prophenoloxidases (proPOs), the enzymes required for melanization. By analyzing the promoter regions of several insect *proPOs*, we identify a conserved GATA/RUNX *cis*-regulatory module that ensures the crystal cell-specific expression of the three *Drosophila melanogaster proPO*. We demonstrate that activation of this module requires the direct binding of both Srp and Lz. Interestingly, a similar GATA/RUNX signature is over-represented in crystal cell differentiation markers, allowing us to identify new Srp/Lz target genes by genome-wide screening of *Drosophila* promoter regions. Finally, we show that the expression of *lz* in the crystal cells also relies on Srp/Lz-mediated activation via a similar module, indicating that crystal cell fate choice maintenance and activation of the differentiation program are coupled. Based on our observations, we propose that this GATA/RUNX *cis*-regulatory module may be reiteratively used during hematopoietic development through evolution.

Keywords: GATA; RUNX; Hematopoiesis; *Drosophila*; Transcription

Introduction

A central question in developmental biology concerns an understanding on how multipotent progenitor cells generate a spectrum of cell types. In this respect, the study of lineage-specific transcription factors is of particular interest, as they establish the gene expression programs intrinsic to cell diversification. *Drosophila* recently appeared as a relevant model in which to characterize the general mechanisms controlling blood cell fate choice and differentiation. Indeed, several developmental strategies and molecular pathways employed during hematopoiesis are conserved from flies to mammals (Evans et al., 2003). In particular, members of the GATA and RUNX transcription factor families, which control several steps of mammalian blood cell development, also participate in *Drosophila* hematopoiesis.

Members of the GATA family contain one or two conserved characteristic zinc fingers and bind the DNA sequence WGATAR (Bresnick et al., 2005). In mammals, three of the six *GATA* genes are reiteratively used from hematopoietic stem cell formation to terminal differentiation into multiple blood lineages (Bresnick et al., 2005). In *Drosophila*, the GATA factor Serpent (Srp) is expressed in blood cell progenitors and its expression is maintained in the two main classes of differentiated hemocytes (Jung et al., 2005; Lebestky et al., 2000; Rehorn et al., 1996): plasmatocytes, which function as macrophages, and crystal cells, which are involved in melanization (an insect-specific defense response). *srp* is required for specification of the progenitors (Mandal et al., 2004; Rehorn et al., 1996) and it also participates in their differentiation (Fossett et al., 2001; Waltzer et al., 2002, 2003).

RUNX transcription factors share a highly conserved 128-amino-acid-long DNA binding domain, the RUNT domain, which recognizes the TGYGGTY consensus sequence (de Bruijn and Speck, 2004). In mammals, all three RUNX factors participate in one or more stages of hematopoiesis. In particular,

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RUNX1, also known as *Acute Myeloid Leukemia 1* (*AML1*), is essential for the development of definitive hematopoietic stem cells (North et al., 1999; Okuda et al., 1996) as well as for lymphocyte and megakaryocyte differentiation in mice (Ichikawa et al., 2004). Also, *RUNX1* is the most frequent target for chromosomal translocation in human leukemia (de Bruijn and Speck, 2004). During *Drosophila* hematopoiesis, the RUNX factor Lozenge (Lz) is necessary for the formation of the crystal cell lineage (Lebestky et al., 2000). Importantly, *lz* function requires the presence of *srp*, and Lz physically and functionally interacts with Srp to induce crystal cell differentiation (Waltzer et al., 2003). Interestingly, the interaction capacity between GATA and RUNX transcription factors has been conserved through evolution (Waltzer et al., 2003) and GATA1 and RUNX1 were shown to cooperate during megakaryopoiesis *in vitro* (Elagib et al., 2003; Xu et al., 2006). Therefore, the cooperation between GATA and RUNX factors plays an important role during hematopoiesis from *Drosophila* to vertebrates.

In *Drosophila*, as in vertebrates, hematopoiesis occurs in two waves. In the first wave, prohemocytes arise from the head mesoderm in the early embryo, whereas in the second wave, blood cells originate from a specialized organ, the larval lymph gland (Holz et al., 2003). Resolution of cell fate choice is best understood during embryogenesis (Bataillé et al., 2005). In the embryo, the differentiation of bipotent prohemocytes into plasmatocytes depends on the partially redundant functions of the transcription factors Glial Cells Missing (Gcm) and Gcm2 (Alfonso and Jones, 2002; Bernardoni et al., 1997). *srp* appears to play a decisive role in plasmatocyte formation because its ectopic expression throughout the mesoderm is sufficient to activate the expression of *gcm* as well as that of several other plasmatocyte markers (Waltzer et al., 2002). On the contrary, ectopic expression of *srp* alone is not sufficient to promote crystal cell formation (Waltzer et al., 2002). Indeed, commitment toward this lineage depends on the activation of *lz* expression in a subset of prohemocytes and only those maintaining *lz* expression differentiate into crystal cells (Bataillé et al., 2005). Furthermore, *lz* expression is continuously required to maintain crystal cell fate (Lebestky et al., 2000). Therefore, it is proposed that commitment to the crystal cell differentiation pathway relies on the maintenance of *lz* expression and on the cooperation between this lineage-specific RUNX factor and the pan-hematopoietic GATA factor Srp (Bataillé et al., 2005). However, no direct target gene of this GATA/RUNX complex has been identified *in vivo*. In addition, how cell fate commitment and differentiation are connected is unknown.

In order to get insights into the molecular mechanisms that control crystal cell development, we decided to characterize direct target genes of the Srp/Lz complex and we analyzed *lz* regulatory regions to uncover how its expression is regulated. We chose to analyze the regulatory regions of the three *Drosophila melanogaster prophenoloxidase* (*proPO*) genes, which are specific crystal cell differentiation markers (Rizki et al., 1985). Comparison of *proPO* promoter sequences in *Drosophila* and other insects allowed us to identify a conserved GATA/RUNX module present in the vast majority of these genes. *Cis*-elements containing this module are sufficient to recapitulate the expression of the three *D. melanogaster proPO* in embryonic and larval crystal cells. Furthermore, we show that both Srp and Lz bind to one of these modules *in vitro* and that the binding of both proteins is required for its activation *in vivo*. In addition, a similar GATA/RUNX module is over-represented in the proximal regulatory region of crystal cell markers and we identified several new Srp/Lz target genes by using this module as a signature for a systematic survey of all *Drosophila* promoter regions. Finally, our results indicate that maintenance of *lz* expression in the crystal cell lineage is also mediated by Srp/Lz-induced transactivation of a similar *cis*-regulatory module. All together, our results suggest that Srp/Lz cooperation through this GATA/RUNX module plays a pervasive role in crystal cell development and ensure the coupling between lineage commitment and differentiation. As the GATA/RUNX interaction is functionally conserved in mammals, a similar module may be reiteratively used during hematopoietic development through evolution.

Materials and methods

Fly stocks

The following *D. melanogaster* lines were obtained from the Bloomington *Drosophila* stock center: *twi-gal4*, *lz-gal4*, *UAS-EGFP*. Lz^{r1}, a null allele of *lz*, was kindly provided by Dr. U. Banerjee. *uas-srp* was previously described in Waltzer et al. (2002). Crosses and embryo collections were performed at 25 °C.

Plasmids and transgenesis

Genomic DNA isolated from wild-type *D. melanogaster* was used to amplify by PCR the putative enhancers of *proPO45*, *proPO54*, *proPO59* and *lz*. The PCR fragments were sequenced and cloned into pCasper-hs43-lacZ vector. Details of the constructs are available upon request. Mutant versions of the *proPO45* and *lz{144}* enhancers were generated with the QuickChange Mutagenesis kit (Stratagene) using oligonucleotides carrying mutations in each RUNX or GATA sites.

For each construct, we established a minimum of three independent transgenic lines by standard P-element-mediated transformation into w^{1118} flies.

Database search

We searched for the presence of a GATA and RUNX sites, in opposite orientation and located within a 100-bp window, in the −500/+100 regions of the 15,800 *Drosophila* promoters listed in the Genomatix database by using the ModelInspector program of the Genomatix suite (http://www.genomatix.de). To build our model, we used the positional weight matrix matrices representing putative binding sites for Srp and Lz described in Senger et al. (2004) and Wildonger et al. (2005), respectively. For each positive promoter, a sequence spanning 50 nucleotides on both sides of the GATA/RUNX signature was used to search for a similar signature in the *Drosophila yakuba* homologous genomic region by BLAST analysis. These criteria identified conserved GATA/RUNX signatures in 266 genes, among which we picked 50 to establish their expression pattern by *in situ* hybridization.

In situ hybridizations and antibody stainings

In situ hybridizations were carried out as previously described (Waltzer et al., 2003). Plasmids containing either the full-length cDNA or an EST were obtained from the Drosophila Genome Resource Center and used to generate anti-sense RNA probes.

The *lz-gal4* enhancer-trap line recapitulates *lz* expression (Lebestky et al., 2000) and was used to label the crystal cells in the embryo and in the larval lymph gland. Double fluorescent immunostaining and *in situ* hybridization were carried out using DIG-UTP labeled *proPO45* anti-sense probe, sheep anti-DIG antibody (1/500) (Roche), donkey anti-sheep conjugated to Alexa Fluor 488 (1/400) (Molecular Probe), rabbit anti-GFP antibody (1/1000) (Biolabs) and goat anti-rabbit antibody conjugated to Alexa Fluor 555 (1/1000) (Molecular Probe). Double fluorescent immunostaining was carried out using rabbit anti-GFP, goat anti-rabbit antibody conjugated to Alexa Fluor 488, mouse anti-β-gal antibody (1/500) (Promega) and goat anti-mouse antibody conjugated to Alexa Fluor 546 (1/400).

Electrophoretic mobility shift assays

Srp, Lz and control lysates were produced using the TNT-coupled reticulocyte lysate system (Promega). Srp and Lz were transcribed from full-length cDNA (Waltzer et al., 2003). For Srp/Lz complex identification, the two proteins were produced in the same reaction. EMSAs were performed by incubating 1 to 3 µl of lysate with ^{32}P end-labeled proPO45{143} probe for 30 min at room temperature in 10 mM HEPES pH 7.9, 50 mM KCl, 1 mM MgCl2, 1 mM EDTA, 5% glycerol and 1 µg poly-d*IdC* in a final volume of 20 µl. For competition experiments, double-stranded oligonucleotides containing either wild-type or mutant GATA or RUNX sites were added to the reaction. The reactions were loaded on a 3.6% polyacrylamide gel, 0.5× TBE and run at 4 °C at 15 V/cm.

Results

Identification of a conserved GATA/RUNX cis-regulatory module in the prophenoloxidase gene family

Phenoloxidases (POs) are enzymes that catalyze the oxidation of tyrosine-derived phenols to quinones (Cerenius and Soderhall, 2004). They serve multiple tasks in insects, including wound healing and melanotic encapsulation of pathogens. In *Drosophila*, crystal cells are the main source of circulating inactive prophenoloxidase (proPO) precursor (Rizki et al., 1985). We previously showed that two of the three *D. melanogaster proPO* (*proPO54*/CG5779 and *proPO59*/CG2952) are expressed in the crystal cells and respond to Srp/Lz-induced activation *in vivo* (Waltzer et al., 2003). Similarly, the third *proPO* (*proPO45*/CG8193) is also specifically expressed in the crystal cell lineage in the embryo and in the larval lymph gland (Figs. 1B and C). Furthermore, we did not detect the expression of any of the three *proPOs* both in *srp* and in *lz* mutant embryos (Supplementary information Fig. 1). Hence, the three *proPOs* are coregulated in the crystal cell lineage and represent likely direct targets for the Srp/Lz complex.

To gain insights into the regulation of the *proPO* family by Srp and Lz, we searched their 5′ flanking regions for the presence of GATA and RUNX binding sites (Fig. 1A). As the PO-dependent melanization reaction is a conserved feature in insects (Cerenius and Soderhall, 2004), we established the phylogenetic relationships between most known insect POs and we analyzed the 5′ flanking regions that we could retrieve from 54 *proPOs* found in 16 insect species (Supplementary information Fig. 2). Remarkably, 47 of these *proPO*s display at least one module composed of a GATA and a RUNX site within a 100-bp window, positioned in opposite orientation, and located less than 500 bp from the transcription start site

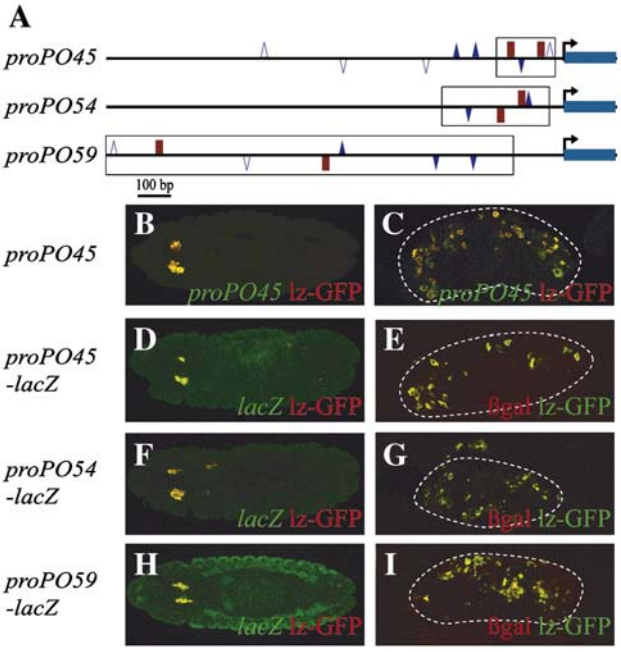

Fig. 1. (A) Organization of the −1000/+100 regions of the three *Drosophila melanogaster proPOs*. Putative binding sites for Srp (blue triangles: WGATAR sites; filled blue triangles: HGATAABV sites corresponding to the Srp-consensus binding sites) and Lz (red rectangles: TGYGGTY sites) are indicated. The genomic regions used to generate the transgenic lines are boxed. (B–I) Endogenous *proPO45* expression (B and C) or *lacZ* expression driven by the *proPO45* (D and E), *proPO54* (F and G) or *proPO59* (H and I) enhancer colocalized with *lz-gal4*-driven *uas-GFP* in the crystal cells. (B, D, F, and H) Merged confocal dorsal view of stage 14 embryos processed to reveal GFP protein and *proPO45* (A) or *lacZ* (D, F and H) transcript. (C, E, G and I) Merged confocal view of third instar larval lymph gland primary lobes processed to reveal GFP protein and *proPO45* transcript (C) or ß-gal protein (E, G and I).

(Supplementary information Fig. 2B). These observations support the hypothesis that GATA and RUNX factors might regulate *proPO* expression in most insects via a conserved regulatory module.

Next, we tested whether the regions comprising this module in the three *D. melanogaster proPO* were sufficient for crystal cell-specific expression. Except for the *proPO59* enhancer that also displays some ectopic activity, *lacZ* expression driven by these three *cis*-regulatory elements is similar to that of the endogenous *proPOs* and colocalizes with *lz*-driven expression of GFP (*lz-gal4,uas-GFP*) in the embryo (Figs. 1D, F and H) and in the lymph gland (Figs. 1E, G and I). Hence, proximal promoter regions containing GATA and RUNX binding sites are sufficient to recapitulate the endogenous *proPOs* expression. These results suggest that the GATA/RUNX module plays a critical role for crystal cell-specific expression of the three *proPOs* in the embryo and in the larva.

Concomitant binding of Srp and Lz to their target enhancer

To further dissect the mechanisms of regulation of the *proPOs* by Srp and Lz, we concentrated on the 143-bp-long *proPO45* enhancer (Fig. 2A). Electrophoretic mobility shift

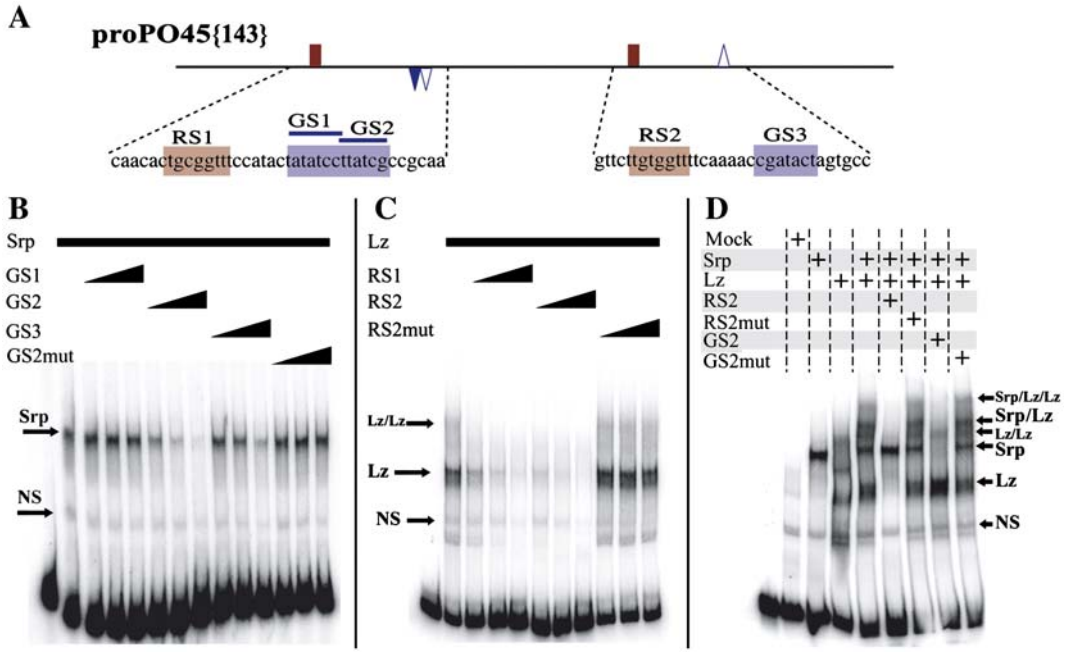

Fig. 2. Srp and Lz bind to the *proPO45* enhancer. (A) Organization of the putative GATA (GS1, GS2 and GS3) and RUNX (RS1 and RS2) binding sites on the 143-bp-long *proPO45* enhancer. (B–D) EMSAs were performed with *in vitro* translated Srp and/or Lz. Increasing concentrations (10-, 50- and 200-folds excess) of unlabeled wild type or mutant oligonucleotides were added to the reaction as indicated in the upper part of each panel. The likely compositions of the different retarded complexes are indicated on the side of each panel. Mock: mock-translated reticulocyte lysate.

assays indicated that *in vitro* translated Srp (Fig. 2B) and Lz (Fig. 2C) bind to this enhancer. The binding of Srp and Lz is specific as it is efficiently competed out by an excess of cold GATA or RUNX oligonucleotides respectively, but not by an excess of oligonucleotides with mutated GATA or RUNX sites. Competition experiments with oligonucleotides spanning individual GATA (GS) or RUNX (RS) sites showed that Lz binds to RS1 and RS2 with similar affinities while Srp displays the highest affinity for GS2, which fits the Srp consensus sequence (HGATAABV) previously defined by SELEX assays (Senger et al., 2004). Srp also binds to GS3 but not to GS1.

We then asked whether Srp and Lz can simultaneously bind this enhancer. When both Srp and Lz were incubated with the probe, we observed two shifts that migrated higher than the shifts observed in the presence of Lz or Srp alone (Fig. 2D). These super-shifts represent Srp and Lz proteins bound to the same enhancer as they are efficiently competed out both by an excess of oligonucleotides containing either a GATA or a RUNX site but not by an excess of oligonucleotides containing mutated GATA or RUNX sites. Therefore, Srp and Lz can simultaneously bind to this enhancer.

Both GATA and RUNX sites are required for Srp/Lz-induced activation

The above experiments suggested that Srp and Lz bind simultaneously to the *proPO45* enhancer to activate its expression in the crystal cells. Accordingly, mutations in either the two Lz binding sites or the two Srp binding sites (GS2 and GS3) completely abolished the *proPO45* enhancer activity *in vivo* (Figs. 3C and D, respectively). Of note, mutations affecting a single Lz binding site or a single Srp binding site did not alter the activity of the enhancer (data not shown). Hence, the number of binding sites or their relative orientation does not appear to be critical. To confirm that binding of Srp and Lz is required and sufficient to activate transcription from the *proPO45* enhancer, we ectopically expressed Lz and/or Srp throughout the mesoderm using the *uas-gal4* system. In line with our previous reports on *proPO54* and *proPO59* (Waltzer et al., 2003), Srp induced a very restricted activation of the endogenous *proPO45* or of the wild-type *proPO45* GATA/RUNX module (Figs. 3I and J), while Lz activated them in the *srp*-expressing domains (Figs. 3E and F). Furthermore, when Srp and Lz were coexpressed, both *proPO45* and *proPO45-lacZ* were strongly activated throughout the mesoderm (Figs. 3M and N). On the contrary, neither Srp or Lz nor Srp/Lz activated the *proPO45* enhancer when the two Srp binding sites or the two Lz binding sites were mutated (Figs. 3G, H, K, L, O and P). Thus, our results show that binding of both Srp and Lz is required and sufficient to activate *proPO45* expression.

A constrained GATA/RUNX signature is the hallmark of Srp/Lz-responsive genes

The occurrence of a conserved GATA/RUNX module in the *Drosophila proPO* genes enticed us to test whether its presence is a more general feature of genes expressed in the crystal cell lineage. 57 genes are reported as being expressed in the crystal cells by the Berkeley Drosophila Genome Project (Tomancak et al., 2002). Re-examination of their expression profile identified 34 of them as *bona fide* crystal cell markers (Supplementary information Table 1). We browsed the promoter regions of these

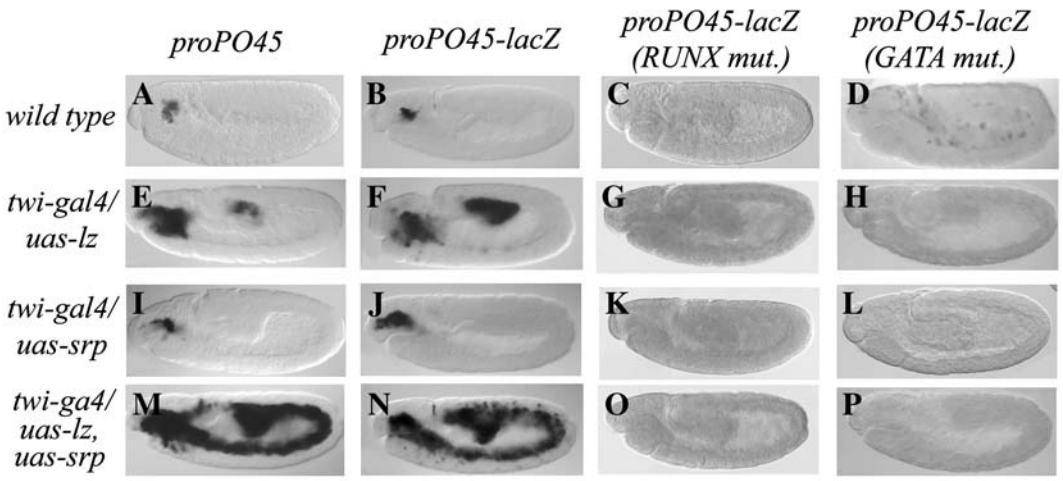

Fig. 3. The binding of both Srp and Lz is required for the activation of the *proPO45* enhancer. (A–P) Side views of stage 10 to 11 embryos processed to visualize *proPO45* mRNA (A, E, I, M), or *lacZ* mRNA driven from the wild type *proPO45* enhancer (B, F, J and N) or from the *proPO45* enhancer carrying mutations in the two Lz binding sites (C, G, K and O) or in the two Srp binding sites (D, H, L and P). Genotypes as indicated to the left of the figure.

genes for the presence of GATA and RUNX sites in opposite orientation within a 100-bp window and observed that 23.5% (or 32.3%) of the crystal cell markers exhibit one or more GATA/RUNX signatures in their −500/+100 (or −900/+100) promoter region. For comparison, the occurrence of this signature in the −500/+100 promoter region of all the *Drosophila* genes is 4.6%. Therefore, the crystal cell-specific promoters display a significant enrichment in this GATA/RUNX signature ($P < 8.4 \times 10^{-6}$; hypergeometric variation). Of note, when the relative orientation of the GATA and RUNX sites is not taken into account, the proportion of positive promoters increased by only 15% (from 23.5% to 27%) for the crystal cell-specific genes against 71% (from 4.6% to 7.9%) for all the *Drosophila* genes, leading to a decreased statistical enrichment ($P < 5.3 \times 10^{-5}$). Thus the presence of closely linked GATA and RUNX binding sites in the proximal promoter region seems to be a characteristic feature of crystal cell-specific genes.

Considering that this GATA/RUNX signature might be a characteristic of Srp/Lz target genes, we searched all the *D. melanogaster* genes that contained such a signature in their proximal promoter region (see Materials and methods). We then systematically searched whether the signature was conserved in *D. yakuba*. Using these criteria, we identified 266 putative Srp/Lz target genes (Supplementary information Table 2) of which we randomly picked 50 for further analysis. By defining their expression pattern in wild-type embryos as well as in embryos ectopically expressing Srp and/or Lz throughout the mesoderm, we categorized these genes in four classes (Table 1). Strikingly, 44% of them might represent Srp/Lz target genes. Indeed, 10 genes were expressed in the crystal cells and responded to Srp/Lz-induced activation (Class I genes, Figs. 4A, E, I and M) and 12 genes were strongly activated upon pan-mesodermal expression of Srp/Lz although they were not detectably expressed in the crystal cells in wild-type embryos (Class II genes, Figs. 4B, F, J and N). Also, we found 4 genes that were activated by ectopic Srp expression although their activation did not appear to be modified by Lz coexpression (Class III genes, Figs. 4C, G, K and O). On the contrary, none of the genes tested seemed to be activated upon ectopic expression of Lz in the absence of Srp. Finally, 24 genes were neither expressed in the crystal cells nor activated by ectopic Srp and/or Lz (Class IV genes, Figs. 4D, H, L and P). In conclusion, our bioinformatic screen proved to be an efficient way to identify genes that might be directly activated by the Srp/Lz complex.

All together, Srp/Lz-mediated gene activation via a GATA/RUNX module may play a central role in crystal cell differentiation by directing the coordinated expression of a large set of genes in this lineage.

Maintenance of lz expression through a functional GATA/RUNX module

In the course of our studies, we identified a GATA/RUNX module in a 1.5 kb upstream element of *lz* that recapitulates *lz*

Table 1
Classification of the 50 genes tested by *in situ* hybridization according to their expression pattern and their response to Srp and/or Lz

Class I Expressed in crystal cells	*Ald*, *CG10602*, *CG11089*, *CG15658*, *CG14269*, *CG6733*, *CG7860*, *mlf*, *oscillin*, *Reg-2*
Class II Not expressed in crystal cells, responsive to Srp/Lz	*CG10725*, *CG13883*, *CG17224*, *CG18745*, *CG31102*, *CG5697*, *CG6475*, *Dot*, *jon65Aiii*, *myo61F*, *Nc73eF*, *Su(Tpl)*
Class III Not expressed in crystal cells, responsive to Srp only	*Cdk4*, *CG16716*, *CG9445*, *RhoGAP88C*
Class IV Not expressed in crystal cells, not responsive to Srp, Lz or Srp/Lz	*CG10650*, *CG11874*, *CG11940*, *CG11943*, *CG12493*, *CG13565*, *CG13836*, *CG14183*, *CG14823*, *CG14956*, *CG1826*, *CG2970*, *CG30421*, *CG31086*, *CG32694*, *CG3332*, *CG3744*, *CG4523*, *CG6640*, *CG7328*, *CG8422*, *CG8944*, *dpr5*, *ets96B*, *par-1*

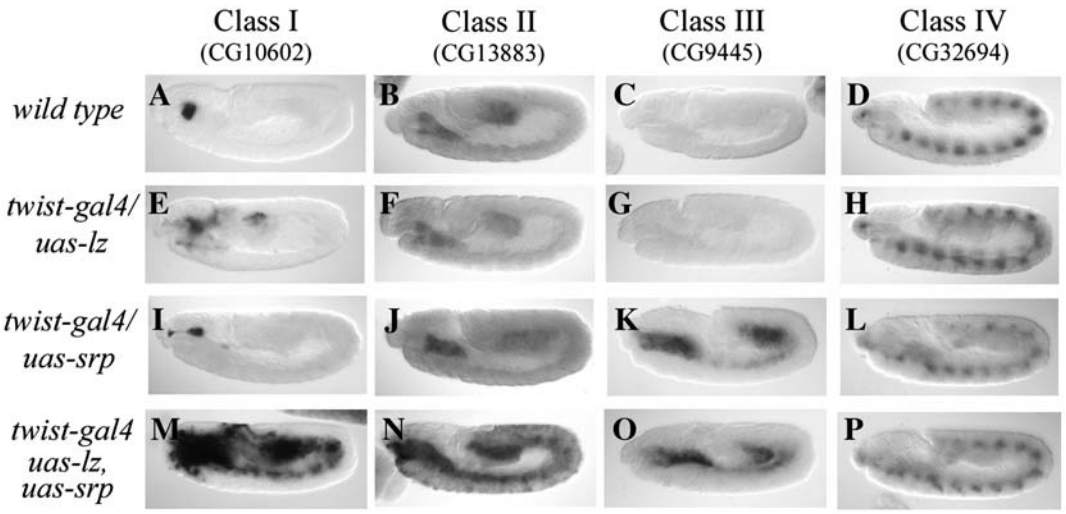

Fig. 4. Examples of expression patterns and responses to Srp and/or Lz of genes containing a GATA/RUNX signature in their promoter. (A, E, I and M) Class I genes were expressed in the crystal cells and responded to Srp/Lz activation. (B, F, J and N) Class II genes were not detectably expressed in crystal cells but were activated in the mesoderm upon overexpression of both Srp and Lz in this tissue. (C, G, K and O) Class III genes were activated by Srp, independently of Lz. (D, H, L and P) Class IV genes were not regulated by Lz and/or Srp. (A–P) Lateral view of stage 11 embryos processed to reveal the expression of CG10602, CG13883, CG9445, or CG32694 as indicated in the upper part of the figure. Genotypes as indicated to the left of the figure.

expression in the crystal cell lineage (Bataillé et al., 2005) (Fig. 5A). Interestingly, a similar GATA/RUNX module is also present in the upstream region of *lz* in other *Drosophila* species (Fig. 5A). A key aspect of crystal cell differentiation is the maintenance of *lz* activity (Lebestky et al., 2000), we wondered whether this module could mediate *lz* maintenance through the activity of Srp/Lz. As shown in Fig. 5, a 144-bp element centered on this GATA/RUNX module is sufficient to drive *lacZ* expression in crystal cells in the embryo (Figs. 5B–D) and in the lymph gland (Supplementary information Fig. 3). Electrophoretic mobility shift assays indicated that Srp and Lz bind specifically respectively to the GATA and RUNX sites present in this enhancer (Supplementary information Fig. 4). Furthermore, the recruitment of both Srp and Lz is required for the activity of this module. Indeed, mutations of the GATA sites or the RUNX sites completely abolished *lz{144}-lacZ* expression in the embryonic and larval crystal cells (Figs. 5F, G and Supplementary information Fig. 3). Finally, the wild-type enhancer was strongly activated by ectopic Spr/Lz expression, whereas mutation of the GATA or the RUNX sites abolished Srp/Lz-induced transactivation (Figs. 5H–P). All together, these results demonstrate that this enhancer is a direct Srp/Lz target and suggest that Srp/Lz directly maintains *lz* expression in the crystal cell lineage via a conserved GATA/RUNX module.

Discussion

Ensuring the stabilization of cell fate choice and cellular differentiation is critical for normal development. Here, we provide evidence that these two steps are intimately coupled during *Drosophila* hematopoiesis. Indeed, our results indicate that both maintenance of the crystal cell fate choice (i.e., maintaining expression of the lineage-specific transcription factor Lz) and the realization of the crystal cell differentiation program rely on cooperation between Lz and Srp.

We identified two direct target genes for Srp and Lz during crystal cell development and showed that both proteins need to bind simultaneously to their respective binding site for these genes to be expressed. In humans, the glycoprotein Ibα and αIIB promoters also contain GATA and RUNX sites and are synergistically activated by GATA1/RUNX1 in cell culture (Elagib et al., 2003; Xu et al., 2006). Therefore, corecruitment of GATA and RUNX transcription factor might be a critical aspect for GATA/RUNX synergistic transactivation. How these factors synergize at the molecular level remains an open question. We did not observe any cooperative DNA binding in our *in vitro* assays. It is possible that simultaneous binding of Srp and Lz allows the formation of a transactivating platform able to recruit efficiently coactivators such as the Mediator or chromatin modifying complexes (Levine and Tjian, 2003). Alternatively, binding of one of the two partners may precede gene activation and poise the enhancer for activation. For example, during mammalian liver development, binding of the albumin enhancer by GATA4 opens a local nucleosomal domain and precedes gene activation, which occurs only upon recruitment of other enhancer binding factors (Bossard and Zaret, 1998; Cirillo et al., 2002). Hence, in the prohemocytes, Srp may already establish the formation of crystal cell-specific chromatin domains primed for activation.

All together, we identified the GATA/RUNX signature in 19 genes expressed in the crystal cell lineage and our results suggest that the Srp/Lz complex directly regulates their expression. Therefore, Srp/Lz induces crystal cell differentiation by coordinating the expression of a large set of genes. Similarly, it has been shown that Srp cooperates with members of the *Drosophila* REL/NFκB family to activate several fat body-specific immunity genes upon infection (i.e., upon nuclear translocation of REL) (Senger et al., 2004). This cooperation depends on closely linked REL and GATA binding sites with the appropriate orientation. In the *proPO45* enhancer, the

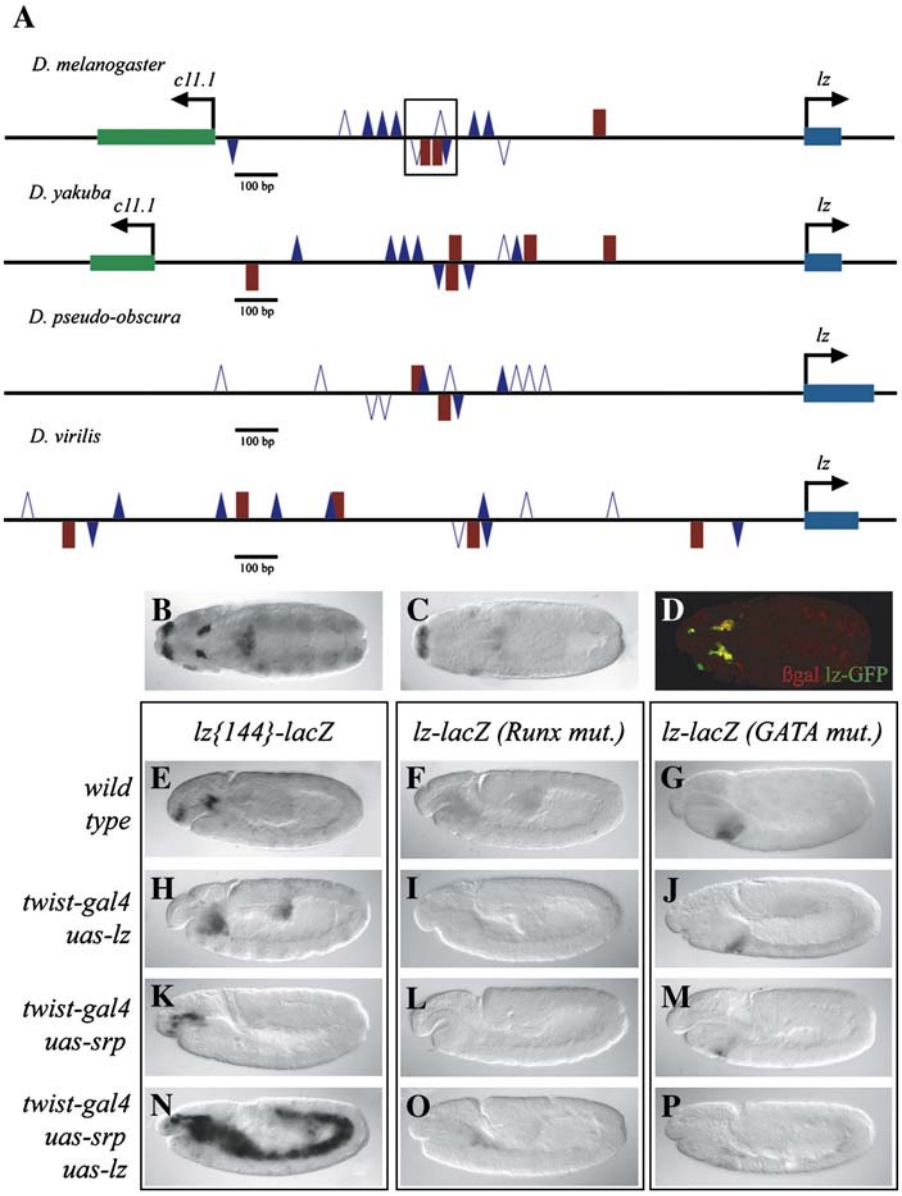

Fig. 5. The Srp/Lz complex directly activates *lz* expression. (A) Schematic representation of *lz* upstream regulatory regions in *D. melanogaster*, *D. yakuba*, *D. pseudo-obscura* and *D. virilis*. Putative binding sites for Srp (blue) and Lz (red) are represented as in Fig. 1. The genomic region used to generate the *lz{144}-lacZ* transgenic lines is boxed. (B–D) Dorsal views of stage 11 embryos. *lacZ* expression driven by the *lz{144}* enhancer is detected in the crystal cell cluster in stage 11 wild-type embryos (B) but absent in a lz^{r1} hemizygous mutant (C), and colocalized with GFP in *lz-gal4,uas-gfp* embryos (D). (E–P) Lateral views of stage 11 embryos processed to reveal *lacZ* expression driven from the wild-type *lz{144}* enhancer (E, H, K and N) or from the enhancer carrying either mutated RUNX sites (F, I, L and O) or mutated GATA sites (G, J, M, P). Genotypes as indicated to the left side of the panels.

opposite orientation between the GATA and the RUNX sites does not appear to be critical in transgenic-based assays. Nonetheless, we found that the opposite orientation of these sites constitutes a conserved characteristic feature of crystal cell-specific genes and thus might be important in their endogenous genomic context. Indeed, long-range DNA sequences play an important role in nucleosomal positioning and the accessibility to these sites might thus be different in their normal environment (Ioshikhes et al., 2006). In line with this hypothesis, the GATA/RUNX signature has been conserved in the vast majority of the *proPO* promoters available from distant insect species. It is tempting to speculate that the regulation of the *proPO* expression by GATA and RUNX factors might represent an ancestral feature. Besides being expressed in the crystal cell lineage (our data as well as Waltzer et al., 2003 and Crozatier et al., 2004), *proPO59* was proposed to be expressed in the lamellocytes in *D. melanogaster* (Irving et al., 2005). Since the transcription factors that control lamellocyte differentiation remains unknown, it could be interesting to identify the regulatory elements required for *proPO59* expression in this lineage. While the function of the *proPOs* in melanization is well established, the functions of the other genes expressed in the crystal cells remain to be elucidated. One of them, *mlf* has an homologue in mammals, which participates in erythro/myeloid

differentiation (Williams et al., 1999) and is found in translocations associated with acute myeloid leukemia in human (Yoneda-Kato et al., 1996). However, the function of *mlf* in *Drosophila* blood cell development is still unknown (Martin-Lanneree et al., 2006).

Our bioinformatic screen, based on the presence of a conserved GATA/RUNX signature in *Drosophila*, allowed us to identify Srp/Lz-responsive genes with a high rate of success. Similar *in silico* screens could help define the repertoire of GATA/RUNX target genes in other species. Notably, the GATA/RUNX interaction is conserved in mammals (Waltzer et al., 2003) and it was shown that human GATA1 and RUNX1 functionally interact during hematopoiesis (Elagib et al., 2003; Xu et al., 2006). In a preliminary analysis, we identified 383 genes that contain a GATA/RUNX signature conserved between mouse and human (L. Waltzer, unpublished data). Strikingly, we observed a statistical over-representation of genes associated to normal and pathological blood cell development in human in this list. In particular, both RUNX1 and RUNX3, but also NFκB (p105), EVI1 (Ecotropic Virus Integration site 1) MLL3 (Mixed Linked Leukemia 3), MKL1 (Megakaryoblastic Leukemia 1), VWF (von Willebrand Factor) and LIF (Leukemia Inhibitory Factor) harbor this conserved signature in their promoter. These observations support the hypothesis that the GATA/RUNX complex plays a critical role in hematopoiesis from *Drosophila* to mammals and suggest that the GATA/RUNX module may be reiteratively used during hematopoietic development through evolution.

Still, the mere presence of the GATA/RUNX signature is not sufficient to provide responsiveness to this complex. For instance, among the 50 genes tested in *Drosophila*, we identified 4 genes that were activated by Srp alone and 16 genes that were not activated by Srp/Lz. Unfortunately, we did not find any particular feature to discriminate between Srp/Lz (or Srp)-responsive and non-responsive genes by simple comparison of their *cis*-regulatory elements. Furthermore, among the 22 genes activated upon ectopic expression of Srp/Lz, 12 were not detectably expressed in the crystal cells. This might reflect either that Srp and Lz are in limiting concentration in the crystal cells or that these genes are expressed in the crystal cells below our detection threshold. Alternatively, these genes might not represent genuine Srp/Lz targets but might be regulated by other GATA/RUNX complexes. There are five GATA and four RUNX in *Drosophila* (Murakami et al., 2005; Rennert et al., 2003; Waltzer et al., 2002), thus other pairs of GATA/RUNX factors might functionally interact to regulate gene expression. Likewise, another GATA, dGATAe cooperates with REL to mediate antimicrobial genes activation in the larval gut (Senger et al., 2006), whereas Srp cooperates with REL in the fat body (Senger et al., 2004). Interestingly, Lz alone regulated none of the genes we tested. All together, these results suggest a model whereby the crystal cell-specific factor Lz must associate with the pan-hematopoietic factor Srp to directly activate the expression of the crystal cell differentiation program.

Finally, we show that the activity of the *lz* crystal cell-specific enhancer also depends on GATA and RUNX binding sites and directly requires the Srp/Lz complex. Given that *lz* function is continuously required to maintain crystal cells (Lebestky et al., 2000), our data strongly suggest that *lz* (and consequently crystal cell) maintenance is achieved through an autoregulatory loop mediated by Srp/Lz. The transition from multipotent hematopoietic progenitors to their differentiated progeny requires the fixation of the initial cell fate choice (Mikkola et al., 2002). Auto-activation of a key lineage transcription factor provides an effective mean to achieve this fixation (Hoang, 2004). All together, our results indicate that in the *Drosophila* bipotent embryonic blood cell progenitors, Srp/Lz-mediated maintenance of *lz* propels both crystal cell fate commitment and differentiation. The occurrence of a conserved GATA/RUNX signature in the mammalian *RUNX1* and *RUNX3* suggests that a GATA/RUNX complex might also regulate their expression during hematopoiesis.

In conclusion, we provide evidence that, in *Drosophila*, the same complex composed of a pan-hematopoietic transcription factor and of a lineage-specific transcription factor is directly involved in maintaining the expression of the lineage-specific partner and in coordinating the expression of a wide array of differentiation markers. At the molecular level, this coupling is achieved through the reiterative use of a similar *cis*-regulatory element. This mechanism ensures a direct connection between cell fate choice and differentiation and might be observed in other developmental contexts from *Drosophila* to mammals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.03.010.

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